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EFFECTS OF TRYPANOCIDAL DRUGS ON THE REPLICATION AND FUNCTION OF KINETOPLAST (MITOCHONDRIAL) DNA IN TRYPANOSOMES

Annual Progress Report

George C. Hill

February 1, 1977

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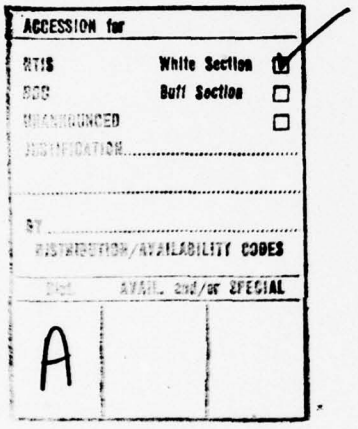

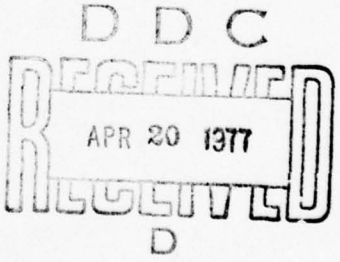

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The purpose of ~~our~~ those studies has been to determine the effects of trypanocidal drugs on the replication of kinetoplast (mitochondrial) DNA. We have also been interested in determining the mode of action of trypanocidal drugs. ~~Our~~ One approach to resolving this problem includes investigating various enzymes in host and trypanosomes, studying the effects of trypanocidal drugs on enzyme systems isolated from trypanosomes and studying the structure and transcription ability of purified kinetoplast DNA. We are interested in determining the reason for the unique selective toxicity of known trypanocidal drugs.

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Our primary results during this last year are:

1. Establishment of procyclic trypomastigotes of T. brucei in vivo;
2. Identification of the electron transport system present during the life cycle of trypanosomes;
3. Demonstration that the trypanocidal drug suramin inhibits the L-α-glycerophosphate oxidase;
4. Determined that berenil has little effect on DNA, RNA and protein levels in berenil-treated T. brucei bloodstream forms;
5. Observed that under optimum conditions, berenil does inhibit RNA synthesis in trypsnosomes but does not show RNA type specificity; or always increases the rate of degradation of RNA.

ABSTRACT

The purpose of our studies has been to determine the effects of trypanocidal drugs on the replication of kinetoplast (mitochondrial) DNA. We have also been interested in determining the mode of action of trypanocidal drugs. Our approach to resolving this problem includes investigating various enzymes in host and trypanosomes, studying the effects of trypanocidal drugs on enzyme systems isolated from trypanosomes and studying the structure and transcription ability of purified kinetoplast DNA. We are interested in determining the reason for the unique selective toxicity of known trypanocidal drugs.

Our primary results during this last year are:

1) Establishment of procyclic trypomastigotes of T. brucei in vivo (22);
2) Identification of the electron transport system present during the life cycle of trypanosomes (22,23);
3) Demonstration that the trypanocidal drug suramin inhibits the L-α-glycerophosphate oxidase (22,27);
4) Determined that berenil has little effect on DNA, RNA and protein levels in berenil-treated T. brucei bloodstream forms;
5) Observed that under optimum conditions, berenil does inhibit RNA synthesis in trypanosomes but does not show RNA type specificity or always increases the rate of degradation of RNA (20).

APPROACH TO THE PROBLEM

Our approach to the problem of developing effective trypanocidal drugs is to study two specific and related areas of the biochemistry of trypanosomes. We are concerned with the regulation and control of the functioning of the electron transport system during the life cycle of trypanosomes. In order to identify targets for potential trypanocides, we must learn more about the properties of the mitochondrion in trypanosomes including:

a) replication of K-DNA;

b) transcription of K-DNA;

c) repression and synthesis of mitochondrial electron transport systems.

In addition we need to study the properties of the α-GP oxidase system. If we can alter the functioning of the mitochondrion or other essential electron transport systems in trypanosomes, we should be able to inhibit the continuation of the life cycle of the trypanosome.

Our working hypothesis is that the synthesis of the mitochondrial cytochrome system or the α-GP oxidase system is essential for the survival of the trypanosomes and under the control of mitochrondial and nuclear DNA. The electron transport systems in trypanosomes could be prevented from functioning by inhibition of the factors which control their synthesis and function. We feel this hypothesis is experimentally testable and pragmatically applicable for chemotherapy.

The overall objective of our research porject is to gain biochemical knowledge on macromolecular synthesis and bioenergetics in African trypanosomes. We want to identify some unique properties in the role of mitochondrial DNA or RNA in the regulation of the functioning of the electron transport system which will provide a tool for inhibiting the replication of the

parasites. In this way, we hope to develop a more rational approach than now exists for the detection of potential trypanocidal compounds.

THE BACKGROUND

One of the major diseases in the world today is trypanosomiasis of man and animals in Africa. It has been placed by the WHO high on the list of the ten major health problems facing mankind, this list including malaria, cancer and heart diseases (1). The importance of trypanosomiasis as a veterinary disease has been well-documented recently in an excellent review by Losos and Ikede (2). The land mass of Africa, south of the Sahara, over which tsetse flies are distributed and that is thus virtually devoid of cattle is estimated to be about 4 million square miles. It has been estimated that if this area could be used, at least 125 million cattle could be raised, and this would more than double the present cattle population of Africa (3).

Our approach to the research on this problem has been to study the molecular biology of the causative agent of the disease - the trypanosome. Trypanosomes are parasitic protozoa of the order Kinetoplastida, Family Trypanosomatidae. These oganisms are characterized by a large amount of mitochondrial DNA (kinetoplast DNA), usually 5-20% of the total cellular DNA which is located within a single, long mitochondrion. These hemoflagellates are the causative agents for several diseases including Trypanosoma cruzi, the causative agent of Chagas' T. gambiense and T. rhodesiense, the causative agent of African sleeping sickness, and Leishmania donovani and Leishmania tropica, the causative agents of visceral and cutaneous leishmaniasis respectively.

We are particularly interested in one organism, Trypanosoma brucei, the causative agent of trypanosomiasis in cattle, sheep and horses. The division of the pleomorphic trypanosomes into three species, T. brucei infecting domesticated animals, and T. rhodesiense and T. gambiense infecting man, has no scientific basis. The human sleeping sickness trypanosomes and their morphologically indistinguishable counterparts in game animals are perhaps best regarded as genetic variants or subspecies of T. brucei. T. brucei brucei of game animals and cattle will not infect man, but T. b. rhodesiense and T. b. gambiense will, causing respectively, acute sleeping sickness in East Africa and a more chronic form of the disease in West and Central Africa. The three T. brucei subspecies are morphologically indistinguishable at all stages in their life cycle.

The question of the function of K-DNA has still not been answered. There are still no publications confirming the transcription of K-DNA in trypanosomes. Several laboratories are investigating the structure of K-DNA but little information is known on its function.

At present, the effects of trypanocidal drugs of K-DNA is difficult to assess. Drugs such as ethidium bromide or acriflavine are known to have many effects on the macromolecular synthesis in cells in addition to binding to DNA. We have shown the effects of acriflavine on the metabolism of C. fasciculata (4). The respiration of dyskinetoplastic organisms obtained by acriflavine treatment is lower than that of normal cells and could be attributed to a decrease in cytochrome content and activity of mitochrondrial enzymes. In addition, we also detected an increase in the activity of a number of dehydrogenases, particularly α-GP and glucose-6-phosphate dehydrogenases (5). However, it must be emphasized that it is certainly not possible to conclusively state that the changes in enzyme activity are a direct consequence of an inhibition of kinetoplast DNA replication.

Simpson and Lasky (6) have reported the isolation from Leishmania tarentolae of two small RNA species, sedimenting at 9 and 12 S in sucrose gradients from a highly purified kinetoplast-mitochrondrion complex fraction. The labeling of this RNA in vivo was sensitive to ethidium bromide (2 μg/ml) but relatively insensitive to actinomycin D or camptothecin. Addition of these RNAs to a wheat germ in vitro protein synthesizing system resulted in a stimulation of incorporation of H-leucine into acid insoluble material. They proposed that these RNA species represent stable mitochondrial messenger RNAs.

The recent findings that the biosynthesis of mitochondrial enzymes is stimulated by agents blocking transcription and translation of mitochondrial DNA suggests that these enzymes are coded by nuclear genes, synthesized on cytoplasmic ribosomes, and transplanted into the mitochondrion. It is clear, therefore, that the functioning of the nuclear genes and mitochondrion are closely related. The cooperation of mitochondrial and nuclear genes specifying the mitochondrial genetic apparatus is essential to the function of this respiratory organelle.

Clearly, our interest in the functioning of the electron transport system in trypanosomes is closely associated not only with the identification of the properties of the α-GP oxidase system or the mitochondrial branched electron transport system, but also associated with the function of the mitochondrial and nuclear genes. Results with Neurospora crassa, an organism with a branched electron transport system, suggests that the nuclear genes coding for mitochondrial enzymes are controlled by mitochondrial protein synthesis. The mechanism of this control is not yet known. Two general possibilities have been proposed:

(1) an indirect mechanism which would involve a metabolic control chain connecting respiration and ATP production in the mitochondrion with nuclear gene expression;

(2) a direct control by a repressor like protein which is coded by mitochondrial DNA, synthesized on mitochondrial ribosomes and exported to the nucleus where it controls the nuclear partner genes for mitochondrial proteins. Numerous examples are now available for role of both nuclear and mitochondrial genes in the synthesis of mito chondrial proteins such as ATPase or cytochrome $\underline{aa}_3$ (7).

Experiments to test these various ideas are needed as we consider the regulation and control of the synthesis of the electron transport systems in trypanosomes.

Identification of mode of action of trypanocidal drugs

The need for new trypanocides cannot be overemphasized. At present, chemotherapy of African trypanosomiasis is dependent on a relatively small number of synthetic drugs. Suramin and pentamidine are used for prophylaxia and treatment of early stages of the disease in man. Organic arsenicals such as tryparsamide and melaminyl compounds are used for advanced cases, when trypanosomes have invaded the central nervous system. The disease in cattle and other domestic animals is controlled by quaternary ammonium trypanocides (antrycide, ethidium, prothidium, and related drugs) and by the aromatic diamidine, berenil. Resistance has been reported to occur against all these drugs and development of resistance to one compound is often accompanied by cross-resistance to another.

In human trypanosomiasis, there is still an urgent requirement for a cheap, simply administered and well-tolerated, preferably "one-shot" drug which would be as effective a prophylactic as pentamidine and active

therapeutically against all stages of the infection in both Gambian and Rhodesian sleeping sickness. It should also be incapable of inducing drug resistance and active also against strains with acquired resistance to other drugs.

Possibly, the two requirements of prolonged tissue retention (for prophylaxis) and ability to penetrate into the central nervous system are mutually exclusive, but with increasing knowledge of the structure and function of the so-called "bloodbrain barrier", this problem should not be insuperable.

In none of the active drugs is the mode of action precisely known. An excellent review of the mode of action of trypanocidal drugs has been prepared by Williamson (8). More recent studies have suggested that berenil and ethidium bromide form complexes with DNA. In the case of eithdium, it is clear this drug is a potent and selective inhibitor of DNA synthesis. It has been shown by several investigators that both phenanthridines and acridines combine with DNA by the heterocylic chromophore of the drug molecules becoming inserted, or intercalated, between the adjacent base pairs in the double-stranded helix of DNA. Such intercalation is achieved by a partial uncoiling of the DNA helix which results in the base pairs above and below the bound drug molecule becoming separated by twice their normal distance (9).

More recently, it has been shown that phenanthridines also bind to supercoiled DNA of the type found in certain tumor viruses, mitochondria of many cell types and kinetoplast of trypanosomes. There is evidence that these drugs bind preferentially to such DNA <u>in vivo</u> and give rise to dyskinetoplast trypanosomes (10) and "petite mutants" of yeast (11). The molecular basis of this preferential binding is not yet fully understood. The findings that have been observed could adequately explain the growth inhibitory

activity of phenanthridine drugs but it remains to be established whether their primary action on bloodstream forms of trypanosomes is to inhibit DNA synthesis.

Berenil, and aromatic diamidine, has been shown under certain conditions to interact with DNA and selectively block kinetoplast replication (12,13). The earliest reported effect observed of berenil is the localization in the kinetoplast of T. brucei. This has been detected by ultraviolet microscopy within an hour of a curative dose being injected intraperitoneally into infected mice and within seconds of the drug being added to an in vitro suspension of trypanosomes (13). Further work has shown that berenil can form complexes with purified DNA, but in contrast to phenanthridines, there is good evidence that the complexes are not formed by intercalation (14).

A detailed examination of kinetoplast DNA isolated from berenil treated T. cruzi has shown that many of the small circular DNA molecules appear as branched structures (12). These forms, which are thought to be replicative molecules, are rarely seen in control preparations, suggesting that berenil does not block the replication of kinetoplast DNA at initiation but binds preferentially to certain specific points in the circular DNA molecule. As for phenanthridines, it cannot be said what is the primary effect of berenil or other diamidines on trypanosomes.

The mode of action of suramin remains enigmatic even after more than a half century of use. In vitro exposure of trypanosomes to suramin at concentrations as low as 10^{-5} M is known to reduce their infectivity whereas concentrations as high as 10^{-2} M do not affect the motility or respiration of cells. As would be expected from its structure, the drug binds avidly to basic proteins and is known to inhibit many isolated enzymes, including hyaluronidase, fumarase, hexokinase, urease, and RNA polymerase (15). The

ready absorption of this drug by plasma proteins may well account for the long retention time of the compound in man and animals and contribute to its value as a prophylactic agent. The question of how a molecule as large as suramin enters trypanosomes is an interesting one and it seems possible that, when protein bound, suramin actively stimulates pinocytosis. As with the other drugs that we have discussed, there is evidence that suramin becomes localized in lysosomes. Again whether this is important to the trypanocidal action of the drug or whether it is a secondary phenomenon is unknown.

An outstanding characteristic of all pathogenic flagellates is the complexity of their life cycles. The changes that occur during their development must all result, directly or indirectly, from changes in gene activity. However, as in other differentiating system, we know little of the mechanisms of gene repression and depression which give rise to parasites able to repond to changing environmental stimuli. Such knowledge should be the goal of future research in this area. It may well provide the basis for a more rational approach to chemotherapy.

RESULTS AND DISCUSSIONS

This contract was awarded on 1 December 1973. Previous results are included in the first annual report which is included in this application and the contract application for 1 January 1976 - 31 December 1976.

Efforts during this year have been devoted to several areas including:

a) Structure of Leptomonas sp. kinetoplast DNA.

b) Cultivation of T. brucei in vitro.

c) Effects of berenil on the DNA, RNA and protein levels in T. brucei.

d) Isolation of mitochondrial RNA polymerases from Leptomonas sp.

e) Effect of berenil on RNA synthesis in T. brucei and Leptomonas sp.

f) Determination of electron transport systems present in trypanosomes.

These experiments have been performed with rats (Rattus norwegicus) Wistar strain and mice (Mus musculus) Swiss - Webster strain.

A. Structure of *Leptomonas* sp. kinetoplast DNA

Using the techniques perfected in this laboratory by Dr. José Remião (16), we have been able to routinely isolate the intact network of kinetoplast DNA from the Leptomonas sp. The geeneration and purification of both covalently close minicircular and full-length linear duplexes of the component DNA of the networks has also become routine.

The minicircles are normally obtained by sonication and sucrose gradient centrifugation, while the linear molecules can be formed through the action of the Eco R1 restriction endonuclease from Escherichia coli or the single-strand specific nuclease S1 purified from Aspergillis oryzae. CsCl-ethidium bromide centrifugation and polyacrylamide gel electrophoresis techniques have been used to preliminarily judge the nature of these forms. We have been able to establish the approximate molecular weight of the unit component minicircle at 550,000 using the electrophoresis and molecular weight standards. This value is quite similar to that established for the minicircles of Leishmania tarentolae (17).

Purified, linear-duplexes generated by S1-nuclease were shown by thermal chromatography on hydroxyapatite (Figure 1) to consist of at least two species of molecules. A minor species was seen to elute around 81 C and a major fraction at about 90 C in 0.12 M phosphate buffer. The G + C content of the main species is about 50% which supports the data established by centrifugation to equilibrium in cesium chloride (16). The complete demonstration of the purity of our preparations and the determination of

molecular weight by comparative contour length measurements awaits our development of the accurate techniques used in electron microscopy. We are currently pursuing this phase of our program.

B. Cultivation of *T. brucei in vitro*

One of our objectives during the past year has been to grow the T. brucei in culture. Using a strain of T. brucei (Lump 1026) obtained from the London School of Tropical Medicine, it has been possible to establish this organism in culture. As seen in figure 2, this strain is pleomorphic, the parasitemia in rats persisting for 30-40 days with recurring parasitemias. In figure 3, one can observe the bloodstream forms from a tail smear of a rat. Primarily, slender trypomastigotes are present. In contrast, figure 4 shows as procyclic trypomastigote which develops in culture.

The kinetics of the establishment of the culture forms can be seen in figure 5. The medium we use has as a base the Joklik-modified Eagle's Essential Medium (Gibco Cat. No. F-13) (see Table 1 and ref. 18). The culture forms became established after 7-10 days in culture. Continued maintenance of the culture produces similar levels of growth (figure 6). These cells have been maintained in culture for 9 months. The culture cells reach a maximum growth of 6-8 x 10^6 cells/ml in 4-5 days.

It is now possible to compare the biochemical differences between the culture (vector) forms and bloodstream forms of T. brucei. This growth will provide the required quantity of cells for our proposed experiments. (See proposed experiment section.)

C. Effect of berenil on the DNA, RNA, and protein levels in *T. brucei*

We have been studying the effects of berenil on the parasitemia of T. brucei in rats. With berenil at 5-10 mg/kg, there is a 50% reduction in

the levels of trypanosomes two hours after treatment. As previously reported, this can be seen in figure 7.

In order to begin to get an idea of what effects berenil is having on the trypanosomes, we have been determining the DNA, RNA, and protein levels in drug-treated cells. Both control and drug-treated cells were purified by DEAE chromatography (19). While berenil has no effect on the separation of the drug-treated cells from the red blood cells, the berenil-treated cells (5 mg/kg) are yellow. Even after thorough washing, berenil is retained in some manner by the trypanosomes. The washed cells (5.0×10^8 trypanosomes/ml) were sonicated in isotonic saline six times for 15 seconds. The homogenate was used to analyze for levels of DNA, RNAS, and protein.

As seen in Table II, in comparison to controls, there is no difference in the levels of DNA and RNA. However, the protein level in the drug-treated cells is two-fold higher than the controls.

These results raise many questions on the mode of action of berenil. The retention of berenil by the trypanosomes and their marked yellow appearance would suggest the drug is bound to the surface coat of the cells. Drug treatment could affect the permeability of the cells. The removal of the trypanosomes from the blood is extremely rapid (e.g., 2-4 hours). Thus, we believe the drug could not be eliminating the trypanosomes by an inhibition of DNA or RNA synthesis.

As the cell numbers decrease, no dead trypanosomes are observed. This observation suggests that there is a rapid lysis of the organisms after drug treatment. In addition, 30-60 minutes after berenil treatment, the cells begin to lose their characteristic movement. They become sluggish, appear to lose a functional undulating membrane and disappear. These results suggest that the cells become a susceptible target for some process

which can lyse or eliminate them rapidly from the blood of the rat. The processes that are involved remain to be determined.

D. Isolation of mitochondrial RNA Polymerase from *Leptomonas* sp.

These experiments have been directed at the identification and characterization of a mitochondrial RNA polymerase. This has been extremely difficult because of the difficulty in isolating a homogenous mitochondrial preparation. In addition, the RNA polymerase activity is not very stable and decreases in activity rapidly.

The scheme for isolation of the Leptomonas sp. mitochondrial RNA is given in figure 8. In recent experiments, the S-30 fraction was centrifuged at 100,000 x g for three hours in order to obtain a high speed supernatant (S-100) and pellet (P-100) fraction. Table III presents the results from such an experiment. There is a consistent 15-fold increase in the S-30 fraction in comparison to the initial mitochondrial pellet. The mitochondrial activity obtained is insensitive to high concentrations of rifampicin (120 µg/ml). Attempts have been made to further fractionate the mitochondiral RNA polymerase activity on DE-52, but these experiments have not been successful.

While it has been possible to demonstrate mitochondrial RNA polymerase activity in Leptomonas sp., the difficulty in isolating a mitochondrial fraction which does not have fused mitochondria will make this research difficult. It is extremely difficult to isolate intact the single mitochondrion present in trypanosomes. We conclude from these results that different experiments are necessary to conclusively demonstrate an RNA transcript from K-DNA.

E. Effect of berenil on RNA synthesis in *T. brucei* and *Leptomonas* sp.

The effect of berenil on the macromolecular synthesis in T. brucei can be investigated in numerous ways. One approach that we have considered is to study the effect of berenil on the incorporation of ^{14}C-labelled uridine into bloodstream forms of T. brucei. In these experiments, trypanosomes purified from DEAE have been employed.

The results for several types of experiments are given in figures 9-11. If added at T_o, berenil has a marked effect on the incorporation of ^{14}C-uridine in T. brucei (figure 9A). Perhaps the uptake of uridine by the trypanosomes is being affected or the urindine is not being converted to an available form. The incorporation of ^{14}C-uridine in the absence of berenil can be observed in this figure also (figure 9A). A chase occurs 20 minutes after the initiation of the experiment. In some experiments, the presence of berenil during the chase increases degradation of synthesized RNA, but these results are not always obtained. A similar experiment with Leptomonas sp. can also be observed (figure 9B).

Berenil does have a marked effect on the incorporation of ^{14}C-uridine into RNA. In figure 10, one can observe the mature species of T. brucei rRNA. The same species of rRNA (e.g., 25s and 18s) that are found in Leptomonas sp. (figure 11) seem to be present. In the presence of berenil (25 µg/ml), the incorporation of ^{14}C-uridine into the rRNA is decreased (figure 10). This is in marked contrast to similar experiments performed with Leptomonas sp. (figure 11B).

These results would suggest that the synthesis and maturation of T. brucei rRNA is more sensitive to berenil than Leptomonas sp.

A problem associated with the above experiments is most easily seen in both the T. brucei and Leptomonas sp. labelling kinetics experiments (figure

9). With both organisms, the incorporation of RNA precursor is only linear for a relatively short period of time. In addition, for T. brucei, labelling in the presence of berenil ceases at about 20 minutes even in the absence of even in the absence of a chase. This result has the effect of superimposing a decreasing rate of RNA synthesis pattern due to a nonexponentially growing culture on the normal pattern of the chase kinetics. It has been most difficult to obtain the optimum incorporation conditions for T. brucei and these procedures will have to be determined. The results as well as problems associated with these experiments are discussed in our recent publication (20). An additional problem of obtaining the bloodstream forms of T. brucei at the same stage of the parasitemia each time will have to be considered.

F. Determination of electron transport systems present in trypanosomes

If we are to consider the effects of trypanocidal drugs on formation and fuction of the electron transport systems in trypanosomes, we must first determine what oxidase systems are present. Efforts have been devoted to characterizing the steady state oxygen kinetics of terminal oxidases in trypanosomes. These experiments have been undertaken with a three-fold purpose:

(1) Characterize the oxidases present during the life cycle of T. brucei;

(2) Identify the mode of inhibition of inhibitors of the α-GP oxidase;

(3) Try to identify new drugs which may inhibit the α-GP oxidase and be effective as chemotherapeutic agents against trypanosomes.

These experiments have been performed with the respirograph system developed by Dr. Hans Degn in Odense, Denmark (21). With this instrument, it has been possible to identify the oxidases present and determine the K_m

for oxygen for the various oxidases present. The open polarographic system is now available in our laboratories for these studies. Our results have recently been submitted to Nature for publication (see manuscripts in appendix, references 22 and 23).

In intact cells, the apparent K_m for O_2 ranges from 2.0 to 8.0 μM (figure 12). The intact cell respiration is not inhibited by CO. However, it is markedly inhibited by salicylhydroxamic acid (SHAM), a known inhibitor of cyanide-insensitive oxidases (24-26). Dixon plots of SHAM inhibition on intact cells reveal an apparent K_i value of 3.3 μM. The SHAM inhibition of the respiration is noncompetitive with respect to oxygen.

The L-α-GP oxidase present in the bloodstream forms has been partially purified by differential centifugation of a cell homogenate. In the presence of α-GP and BSA, the partially purified oxidase has an apparent K_m for O_2 = 2.1 ± 0.5 μM (figure 13). In the absence of BSA, the V_{max} and K_m are decreased 4-6 fold. The reciprocal plots in the presence and absence of BSA are parallel, demonstrating an activation of a component of the α-GP oxidase system by BSA (Figure 14). The partially purified L-α-GP oxidase is markedly inhibited by SHAM, this inhibition being noncompetitive with respect to O_2 with a K_i = 5.4 ± 0.3 μM SHAM (figure 15). SHAM reacts with the oxidized form of the enzyme. Its inhibition of the oxidase increases as the time of its exposure to the oxidized form of the enzyme increases. Incubation of the reduced form of the enzyme with the inhibitor has little or no effect. As the enzyme concentration is increased, the inhibition by SHAM is reversed.

While the use of SHAM has permitted us to identify a specific inhibition for this alternate oxidase, we have also been studying the effect of the trypanocidal drug suramin on the L-α-glycerophosphate oxidase. We have

determined that suramin is an uncompetitive inhibitor with respect to oxygen with a K_i 8.0 ± 1.0 μM suramin (figure 16). Thus, this trypanocidal drug is an extremely sensitive inhibitor of the L-α-GP oxidase. Further experiments on this new observation will be detailed in the proposed experiment section.

We have also studied the oxidases present in procyclic trypanomastigote culture forms of T. brucei. The reciprocal plots of the established culture forms of T. brucei reveal an apparent K_m = 0.1 ± 0.02 μM O_2, suggesting that the affinity for O_2 of the oxidases present in these forms is very high (figure 17). In addition, steady state studies with various inhibitors added reveal that at least three different oxidase systems are present (figure 18).

(1) An azide-insensitive, SHAM-sensitive oxidase, the α-GP oxidase;

(2) An azide-sensitive, SHAM-insensitive oxidase, cytochrome aa_3 with a low apparent K_m for O_2;

(3) An azide-insensitive, SHAM-insensitive oxidase with a low apparent K_m for O_2.

The last two oxidases are inhibited by CO.

There are several respiratory systems present in trypanosomes during their life cycle and they have different affinities for O_2. In the bloodstream forms, the α-GP oxidase has a high apparent K_m for oxygen. This would suggest that the oxidase has a low affinity for O_2. This oxidase accounts for at least 95% of the respiration of bloodstream forms.

After bloodstream trypanosomes are transferred to culture, additional oxidase systems develop. These oxidases have a very low apparent K_m for oxygen and most likely have high affinities for oxygen. It is clear that an azide-sensitive oxidase, cytochrome aa_3, is present. This oxidase accounts

for at least 50% of the cell respiration. In addition, an azide and SHAM-insensitive oxidase is present. This oxidase accounts for 25-35% of the cell respiration and could be cytochrome o. Both these oxidases are sensitive to CO. A third oxidase, SHAM sensitive, azide-insensitive, is evident and is probably the α-GP oxidase which persists from the bloodstream stages.

During the transformation of T. brucei bloodstream forms to culture forms, it appears that an oxidase with a high affinity for oxygen is required. With the oxygen tension extremely low in the midgut of the tsetse fly, in comparison to the bloodstream of the vertebrae, the synthesis in the trypanosome of oxidases with high affinities for oxygen may be essential during transformation. The factors that regulate the synthesis and function of these oxidases during the life cycle of trypanosomes is a fascinating question which remains to be answered. A more detailed description and discussion of the electron transport system in trypanosomes can be found in a recent review prepared by the principal investigator (27).

CONCLUSIONS

During the past year, we have continued our efforts on determining the effects of trypanocidal drugs on several related aspects of the function of trypanosomes. Our major efforts and results this past year have been:

a) Establishment of procyclic trypomastigotes of T. brucei in vivo (22);

b) Identification of the electron transport system present during the life cycle of trypanosomes (22,23);

c) Demonstration that the trypanocidal drug suramin inhibits the L-α-glycerophosphate oxidase (22,27);

d) Determined that berenil has little effect on DNA, RNA and protein levels in berenil-treated T. brucei bloodstream forms;

e) Observed that under optimum conditions, berenil does inhibit RNA synthesis in trypanosomes but does not show RNA type specificity or always increases the rate of degradation of RNA (20);

f) Recognized the difficulties involved with pulse-chase kinetic experiments using T. brucei.

RECOMMENDATIONS

Our major recommendation is concerned with the development of a system which would permit the characterization of the bloodstream forms after growth in vitro. It would facilitate the testing of drugs for typanocidal drug activity, the characterization and elucidation of the mechanism of antigenic variation and the labeling of bloodstream forms of trypanosomes with radioactive precusion of macromolecules if an in vitro system could be developed.

Another area of increasing interest is the L-α-glycerophosphate oxidase present in bloodstream forms. This oxidase is inhibited by the trypanocidal drug suramin. It is important to determine if this oxidase can be a specific target for trypanocidal compounds. In addition, trypanosomes survive anaerobically and the identification of the alternate pathway is most important to the development of chemotherapy of trypanosomiasis.

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APPENDIX

TABLE I

Modified Minimal Essential Medium
for Hemoflagellates (F-13)

	Per Liter
Minimum Essential Medium (Joklik-modified) cat. no. F-13 with: L-Glutamine, Antibiotics and $NaHCO_3$ without: Calcium Chloride (GIBCO)	13.4 gm
MEM Amino Acids 50X (GIBCO)	10.0 ml
MEM Non-Essential Amino Acids 100X (GIBCO)	10.0 ml
Na-pyruvate 100X (GIBCO)	11.0 ml
Biotin	0.1 mg
Hemin solution (1 mg/ml in pyridine)	6.0 ml
Fetal Calf Serum (GIBCO)	50.0 ml
HEPES*	6.0 gm
L-Proline	1.7 gm

pH to 7.2-7.4 and filter sterilize

*Final concentration of buffer = 0.025M

TABLE II

Levels of DNA, RNA and Protein in
Control and Berenil-treated *T. brucei*

	DNA*	RNA*	Protein*
Control	0.98±0.5	2.3±0.7	16.6±5.4
Berenil-treated (5 mg/ml)	0.89±0.3	2.3±0.9	30.5±7.6

* The concentration is expressed as $\mu g/10^7$ cells

TABLE III

Isolation of Mitochondrial RNA Polymerase

Activity from Leptomonas sp.

Function	Specific Activity*	Purification Fold
High speed Pellet	11.8	1
Sonicated Pellet	28.9	
S-30	184.7	15.6
S-100	173.9	14.7
P-100	132.3	11.2

* p moles of ^{3}H-UTP incorporated per mg protein for 10 min. at 37° C.

TABLE IV

EFFECT OF SURAMIN ON INFECTION OF

Trypanosoma brucei

CELL NUMBERS

Hours after Treatment	Control	Suramin (5.0 mg/kg)	Suramin (10.0 mg/kg)
0	3.7×10^8	7.2×10^8	4.6×10^8
+2 hrs.	4.7×10^8	6.8×10^8	4.6×10^8
+5 hrs.	7.0×10^8	6.6×10^8	4.1×10^8
+19 hrs.	Dead	6.8×10^6	0
+26 hrs.		0	0

FIGURE LEGENDS

Figure 1. Thermal chromatography of kinetoplast DNA of Leptomonas sp. on hydroxyapatite in 0.12 M phosphate buffer. Network forms of kinetoplast DNA were treated with S1-nuclease. Then linear-duplex molecules were obtained by chromatography through Bio-Gel A-5 M and applied to the hydroxyapatite column at 60 C.

Figure 2. Parasitemia curve of rat infected with T. brucei (Lump 1026). Note the fluctuations in parasitemia. The inoculum was 1×10^6 cells. The rat died on day 45.

Figure 3. Photograph of bloodstream forms of T. brucei (Lump 1026) from an infected mouse. Note the position of the kinetoplast in these slender forms. Magnification 500X.

Figure 4. Photograph of established culture forms of T. brucei (Lump 1026). The cells were grown in a Joklik modified Eagle's minimal medium (5). Note the position of the kinetoplast. These are procyclic trypomastigotes. Magnification 400X.

Figure 5. Kinetics of cell growth during the transformation of T. brucei (Lump 1026) from bloodstream form to culture forms. 200 ml of culture media were inoculated with 4.0 ml of rat blood containing 2.0×10^8 cells.

Figure 6. Growth of the culture forms of T. brucei (Lump 1026) over 10 successive generations. The generation time was approximately 18 hrs.

Figure 7. Effect of berenil on the parasitemia of T. brucei. Three rats were treated when the infection reached a level of 5.0×10^8 cells/ml. The three control rats died at $1.4 - 1.6 \times 10^9$ cells/ml.

Figure 8. Scheme for isolation of mitochondrial RNA polymerase from mitochondrial fraction of Leptomonas sp.

Figure 9A. The effect of berenil on (^{14}C) uridine incorporation in T. brucei. At T_0 (^{14}C) uridine (0.25 uC; 54 mC/mmole) was added to DEAE purified T. brucei in buffer at 37°C. At the times indicated 200 µl samples were taken for radioactivity incorporation measurements. At 21 min (arrow), excess unlabeled uridine was added to part of the culture. (●) no chase; (○) uridine chase; (■) uridine chase plus berenil (20 µg/ml); (□) berenil added at t_0 and uridine chase.

Figure 9B. The effect of berenil on (^{3}H) uridine incorporation in Leptomonas sp. Experimental protocol was similar to Fig. 8A but with Leptomonas sp. suspended in semi-defined medium at 27°C. (●) no chase; (○) uridine chase; (■) uridine chase plus berenil (20 µg/ml); (□) berenil at t_0 plus uridine chase.

Figure 10. Effect of berenil on synthesis of RNA in T. brucei. DEAE purified T. brucei, resuspended in buffer to 10^8 cells/ml, were labeled for 40 min with ^{14}C-uracil (5 µC; 62 mC/mmole) either in the absence (solid lines) or presence of berenil (dashed line) at 25 µg/ml. Samples were analyzed by sucrose density gradient centrifugation.

Figure 11A. Synthesis and maturation of Leptomonas sp. + rRNA. Cultures (5 ml) of Leptomonas sp. in semi-defined medium were labeled for various times with (^{3}H) uridine (100 µC; 28 C/mmole). Samples were analyzed by sucrose density gradient centrifugation.

Figure 11B. Effect of berenil on synthesis and maturation of Leptomonas sp. rRNA. Cultures of Leptomonas sp. were labeled for approximately 4 generations with (^{14}C) uracil (5 mC; 62 mC/mmole), concentrated, washed, and pulsed for 20 min with (^{3}H) uridine (100 μC; 28 c/mmole). Analysis of RNA was as in Fig. 11A. (solid lines) (^{3}H); (dashed lines) (^{14}C).

Figure 12. Reciprocal plots of DL-α-glycerophosphate oxidation against low oxygen concentration with bloodstream forms of T. brucei. Cells or enzyme were added to a reaction mixture of 40 mM KCl, 40 mM Tris HCl, 8 mM $MgCl_2$, 1.6 mM EDTA, 10 mM glucose, 10 mM DL-α-GP and 0.02% BSA. The three curves (a, b, and c) represent increasing concentrations of cells (1.0, 1.5, and 2.0 × 10^7 cells/ml).

Figure 13. Reciprocal plots of DL-α-glycerophosphate oxidation against oxygen concentration with the α-glycerophosphate oxyidase from bloodstream forms of T. brucei. The trypanosomes were separated from the red blood cells of infected rats using the DEAE procedure of Lanham (2). The cells were washed in isotonic buffer and then suspended (2 × 10^8 cells/ml) in a hypertonic buffer consisting of 2 mM Tris, 0.2 mM EDTA-pH 7.5. The cells were broken by passage 3X by hand pressure through a #25 gauge needle at 4 C. This procedure yielded 90-95% breakage of the cells. Sucrose was added to a final concentration of 0.25 M and the homogenate centrifuged initially at 1085 × g for 10 minutes. The supernatant was recentrifuged at 39,100 × g for 10 minutes and washed with isotonic buffer. This high speed pellet was used in the enzyme studies. The three curves (a, b

and c) represent increasing concentrations of enzyme (4.0, 6.0, and 8.0 mg protein/ml).

Figure 14. Reciprocal plots of T. evansi L-α-glycerophosphate oxidase activity against low concentrations of oxygen in the absence and presence of bovine serum albumin (BSA). Enzyme concentration is 4.0 mg protein/ml.

Figure 15. Dixon plot for SHAM inhibition of α-GP oxidase isolated from T. brucei.

Figure 16. Reciprocal plot of T. evansi α-GP oxidase actitivty against low concentration of oxygen in the presence of increasing concentrations of suramin.

Figure 17. Reciprocal plots of culture forms of T. brucei DL-α-glycerophosphate oxidation against low concentrations of oxygen. The three curves (a, b, and c) represent increasing concentrations of cells (3.0, 4.5 and 6.0×10^7 cells/ml).

Figure 18. Steady state oxygen trace of established T. brucei culture cells. The system is as previously described (4). The concentrations given are the final concentration of additions in the cuvette. The concentration of cells was 1.2×10^8 cells/ml.

Figure 1

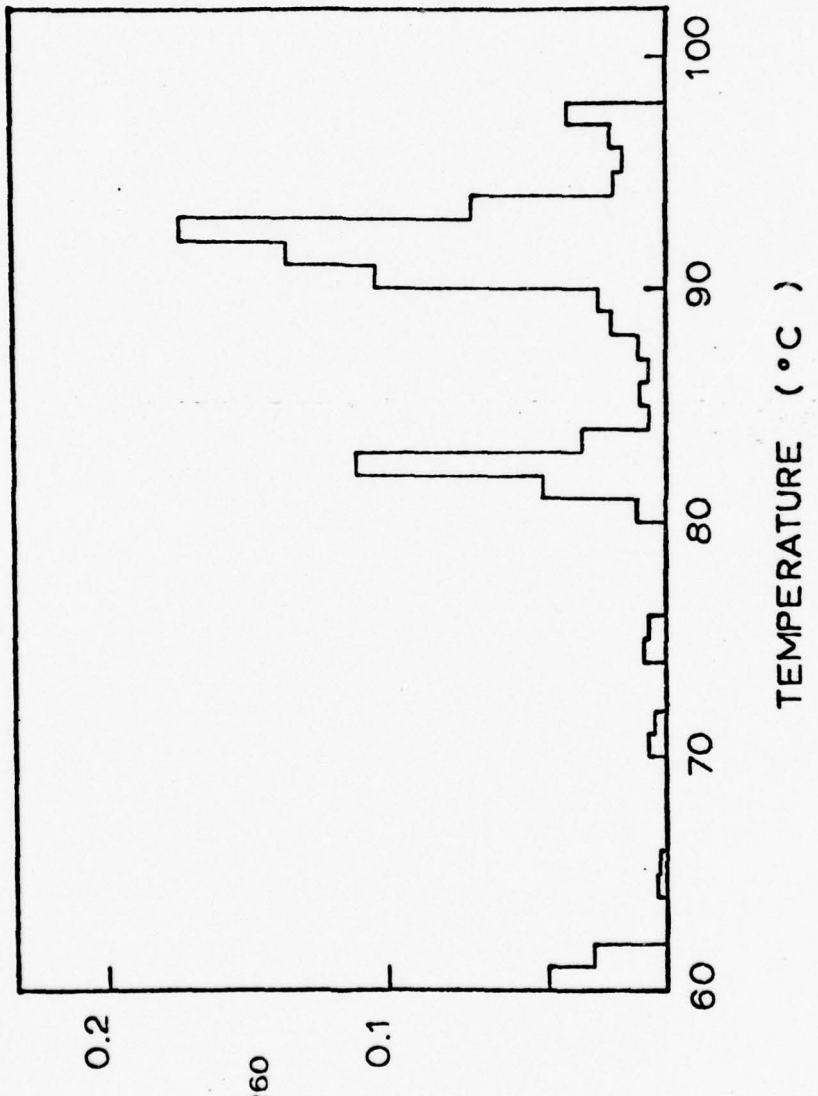


Figure 2

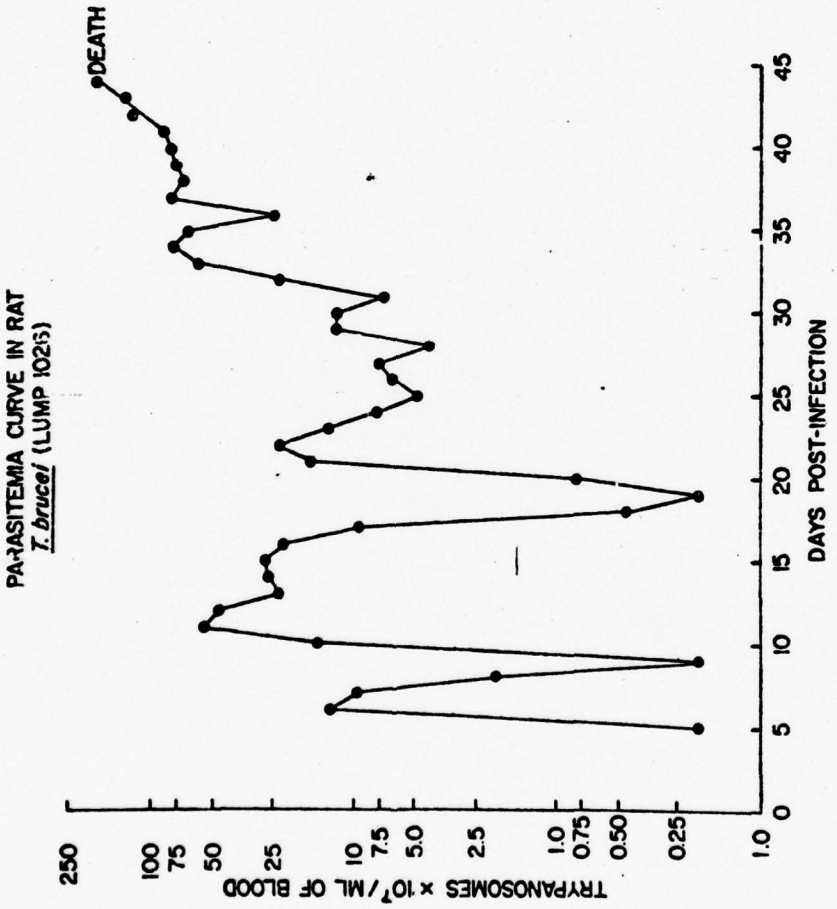

PARASITEMIA CURVE IN RAT
T. brucei (LUMP 1021S)
DEATH
TRYPANOSOMES x 10⁷/ML OF BLOOD
250
100
75
50
25
10
7.5
5.0
2.5
1.0
0.75
0.50
0.25
1.0
0
5
10
15
20
25
30
35
40
45
DAYS POST-INFECTION

Figure 3

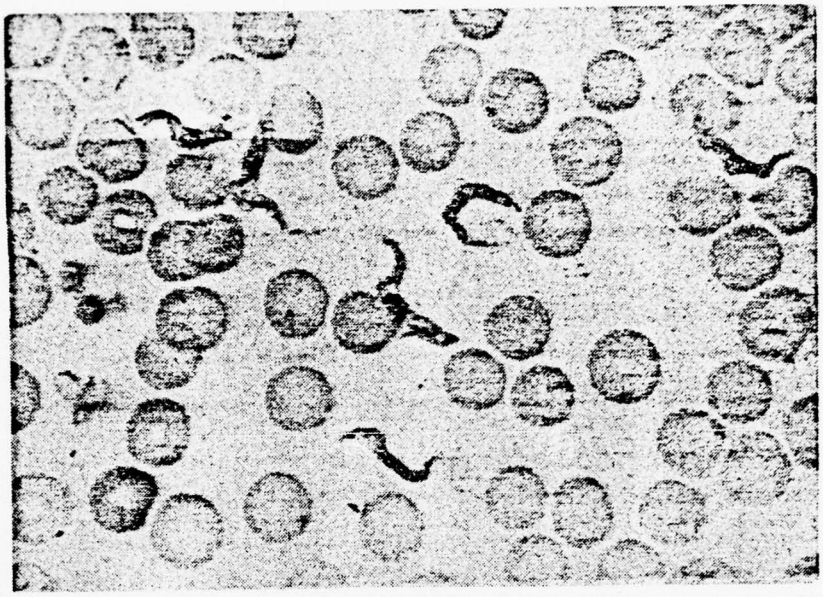


Figure 4

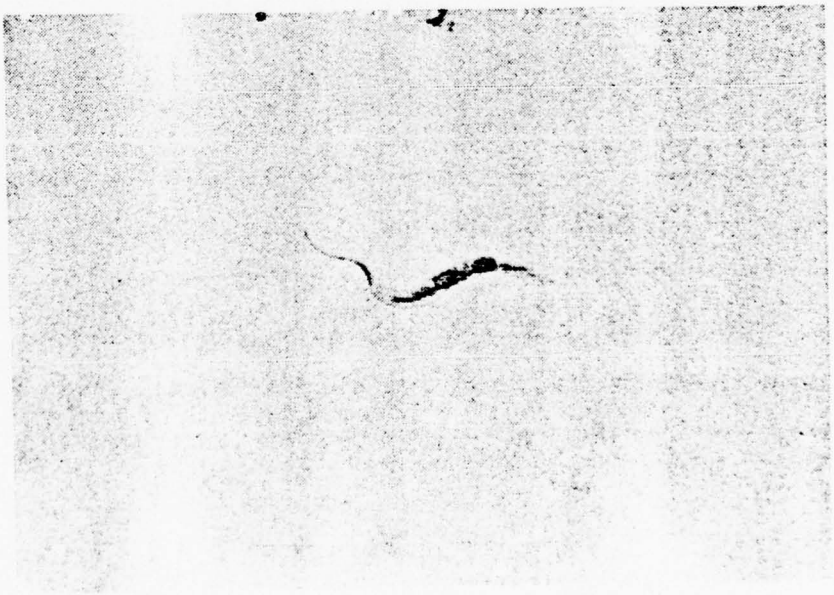


Figure 5

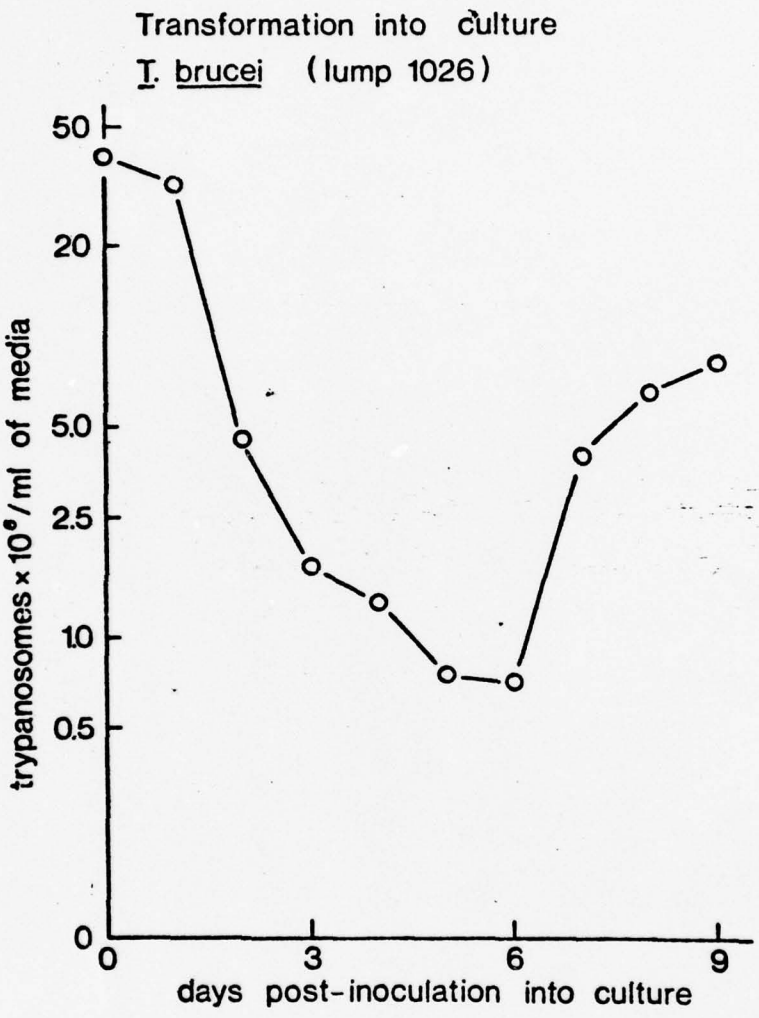

Transformation into culture
T. brucei (lump 1026)
50
20
5.0
2.5
1.0
0.5
0
trypanosomes x 10⁶/ml of media
0
3
6
9
days post-inoculation into culture

Figure 6

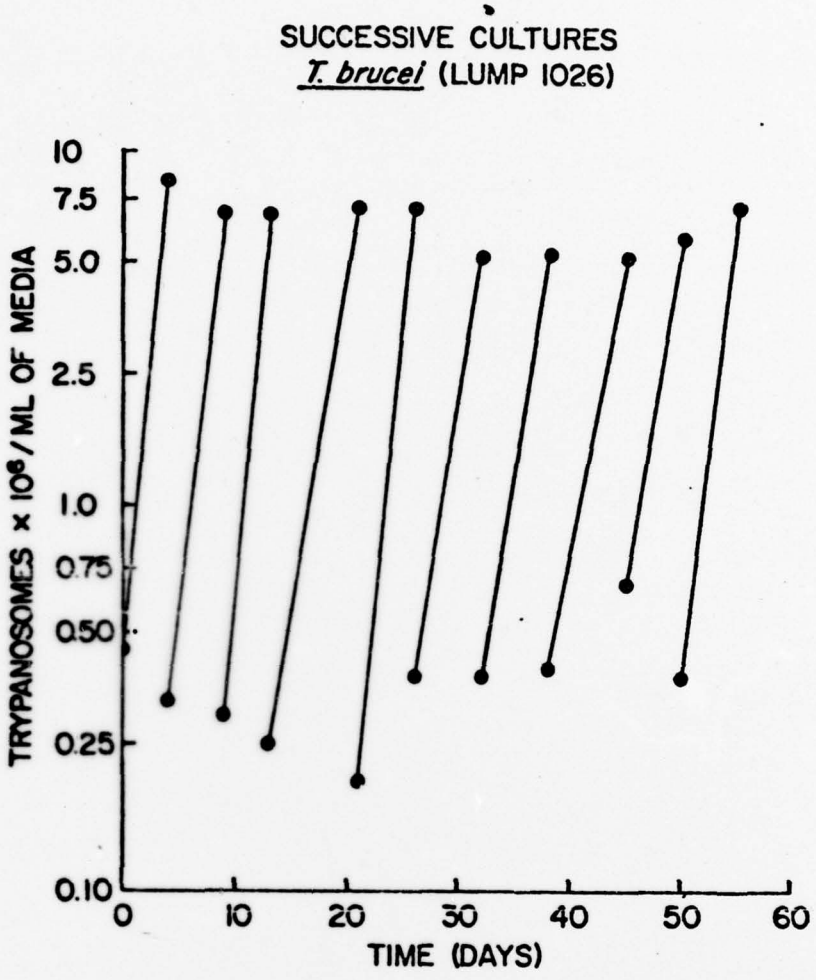

SUCCESSIVE CULTURES
T. brucei (LUMP 1026)
TRYPANOSOMES x 10⁶/ML OF MEDIA
10
7.5
5.0
2.5
1.0
0.75
0.50
0.25
0.10
0
10
20
30
40
50
60
TIME (DAYS)

Figure 7

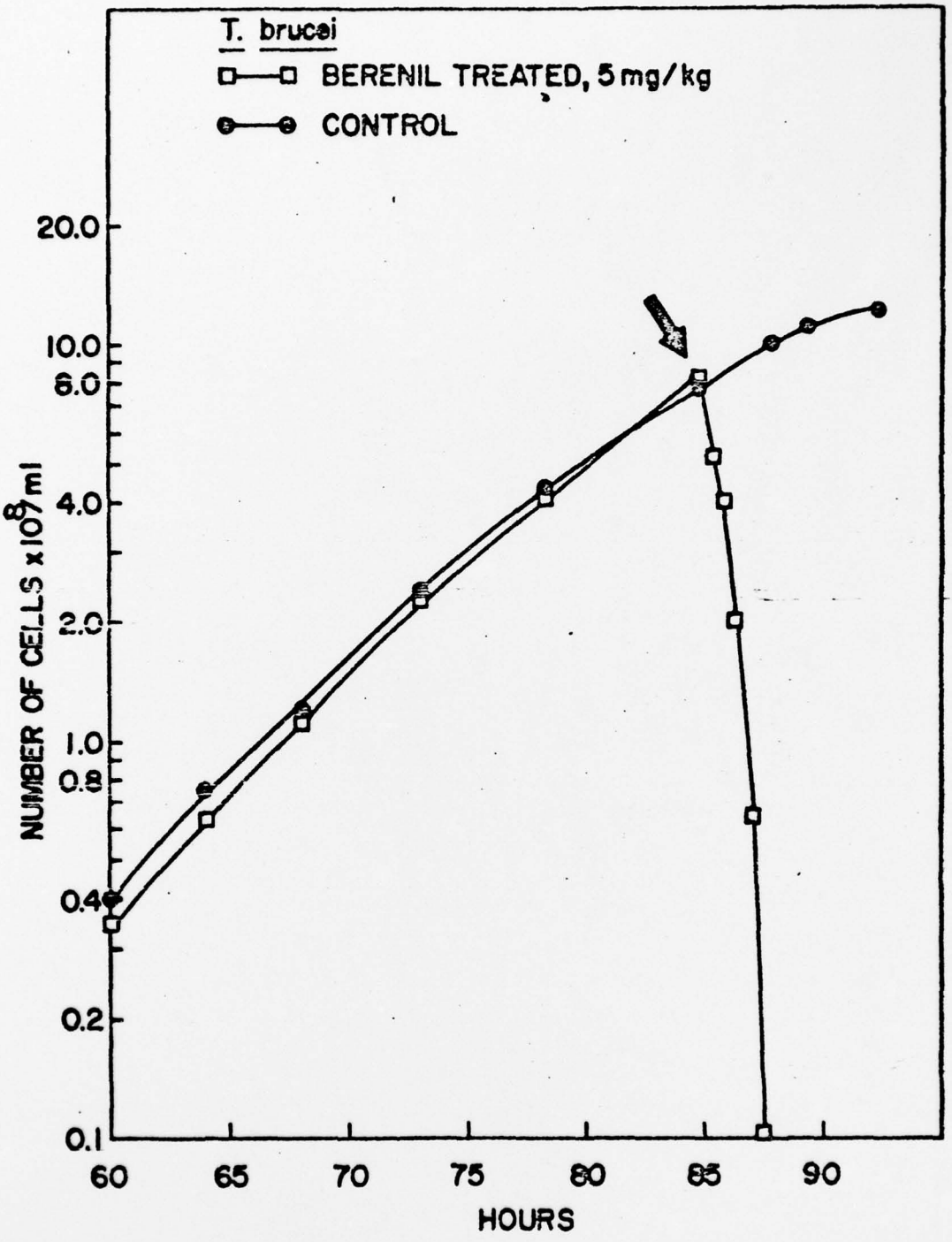

T. brucei
BERENIL TREATED, 5 mg/kg
CONTROL
NUMBER OF CELLS x 10^8/ml
20.0
10.0
6.0
4.0
2.0
1.0
0.8
0.4
0.2
0.1
60
65
70
75
80
85
90
HOURS

Figure 8

Isolation of Mitochondrial RNA Polymerase Activity from Leptomonas sp.

Harvest cells (2.5 - 3.0 X 10^7 cells/ml)

↓

Wash cells with STED + PMSF

↓

Resuspend in STED + PMSF to a cell concentration of 6 X 10^8 cells/ml

Lyse cells by passing through 26½ g needle 3 times at 100 psi.
Add 60% sucrose immediately to a final concentration of 0.2 M

↓

Centrifuge at 3000 rpm for 3 min. (Sorvall. SS-34)

Pellet (discard)	Supernatant

↓

Centrifuge at 11,000 rpm for 10 min (SS34)

Pellet	Supernatant (discard)

↓

Wash pellet with STED + PMSF

↓

Resuspend in STED + PMSF

↓

Sonicate for 20 sec. at maximum setting microtip at 10 sec. interval.

↓

Centrifuge at 30K x g (16 K rpm, SS34) for 30 min.

Pellet (discard)	Supernatant (S-30)

↓

Further Purification

Figure 9

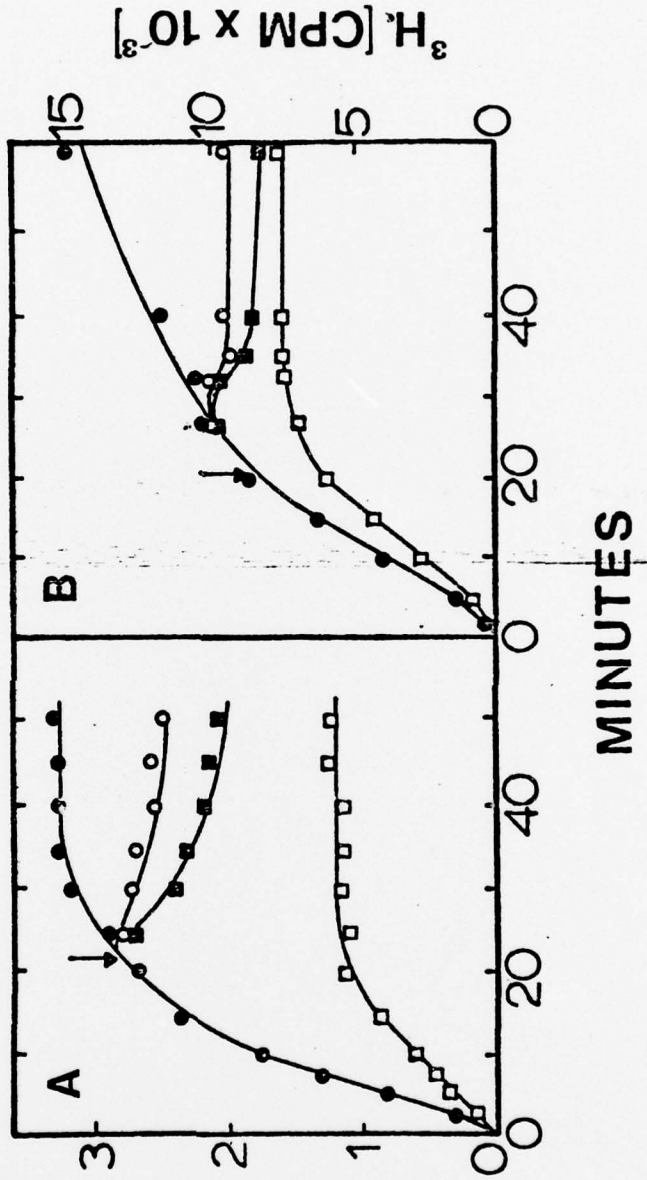


Figure 10

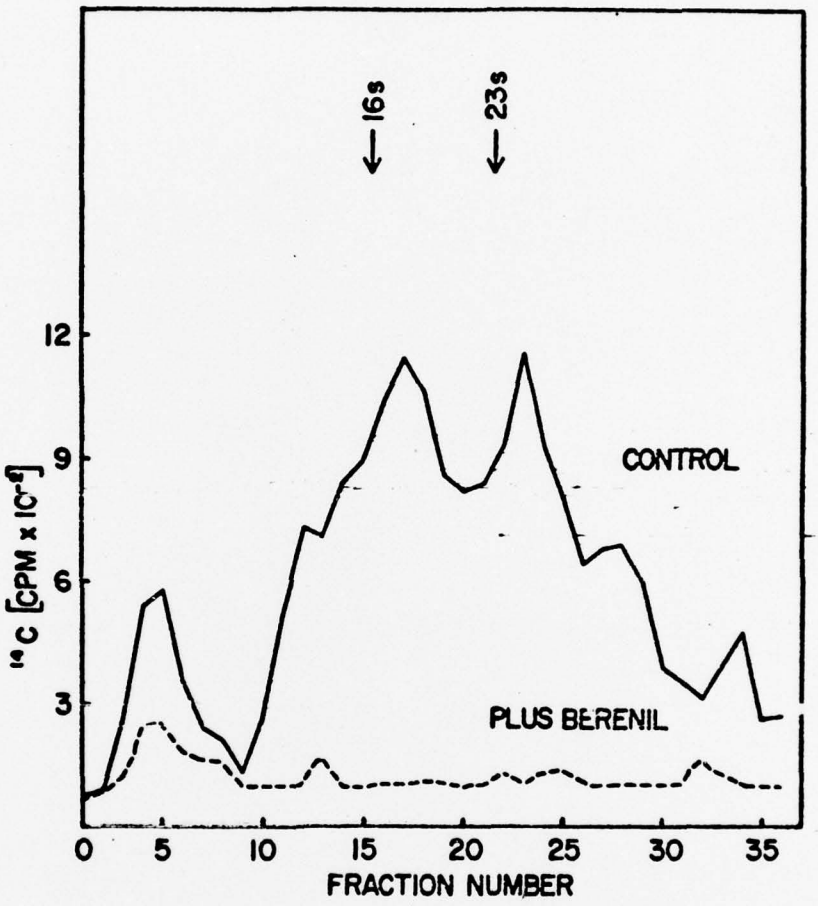

16s
23s
12
9
6
3
^{14}C [CPM x 10^{-4}]
CONTROL
PLUS BERENIL
0
5
10
15
20
25
30
35
FRACTION NUMBER

Figure 11

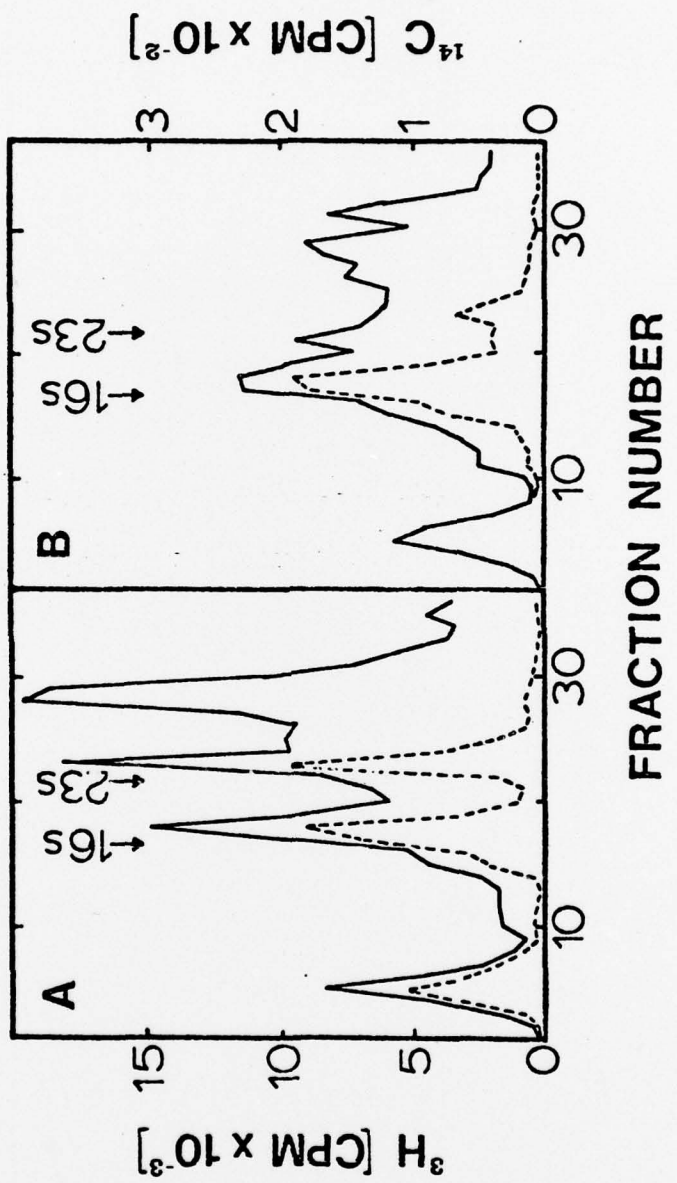

^{14}C [CPM x 10^{-2}]
3
2
1
0
B
16s
23s
30
10
FRACTION NUMBER
A
16s
23s
30
10
15
10
5
0
^{3}H [CPM x 10^{-3}]

Figure 12

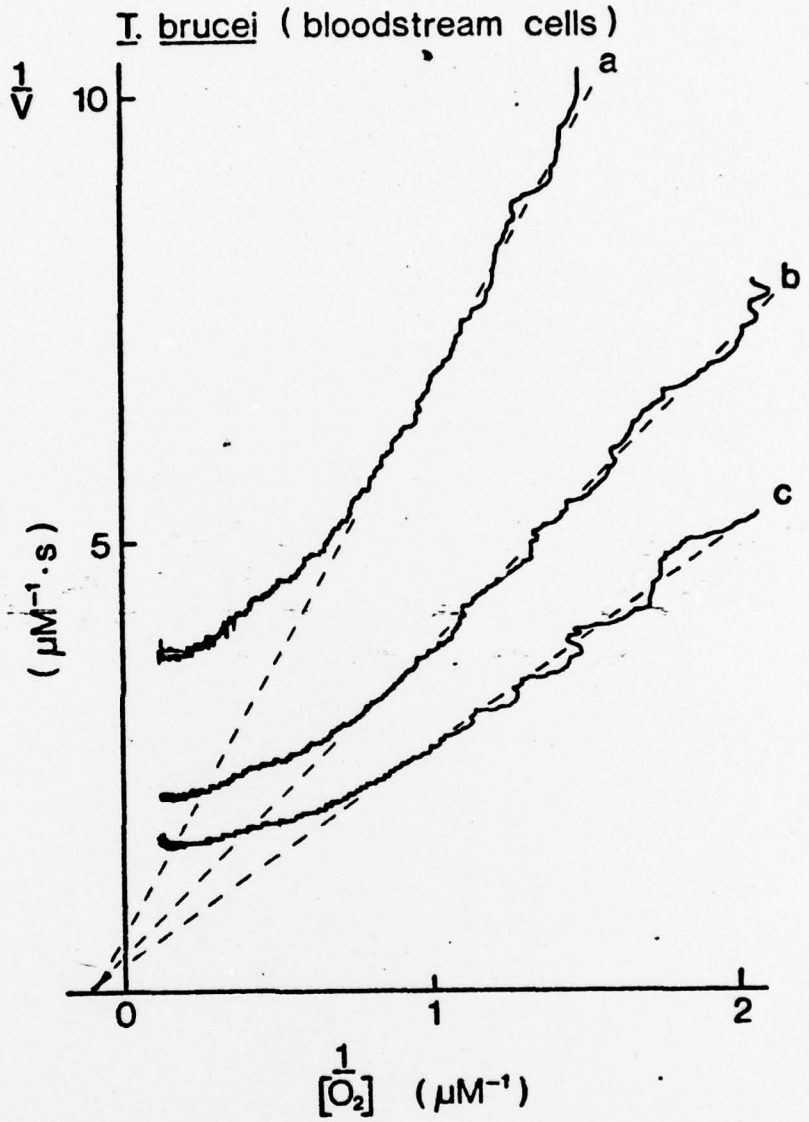


Figure 13

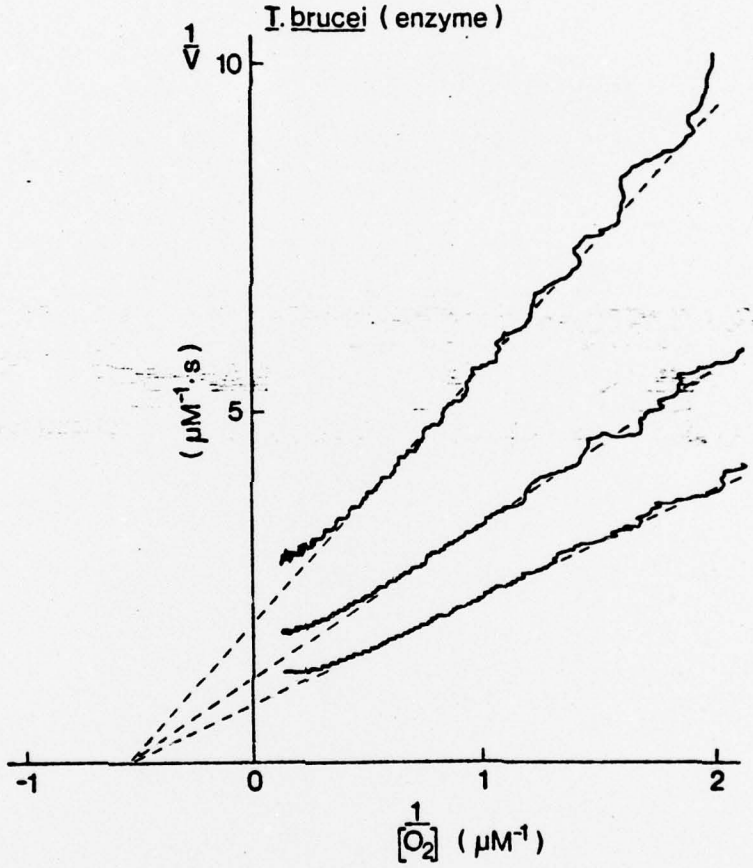


Figure 14

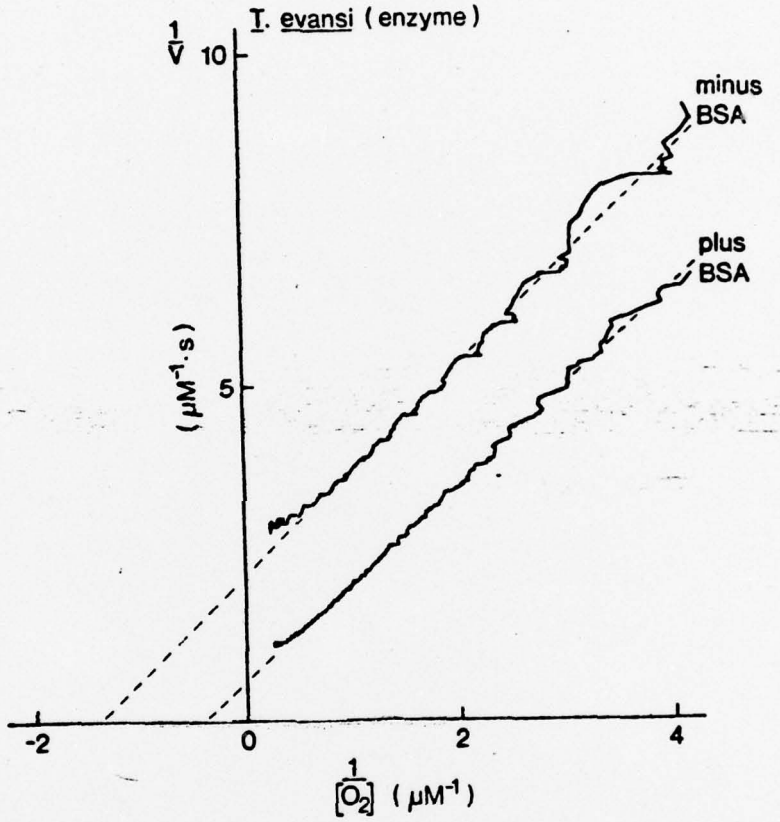


Figure 15

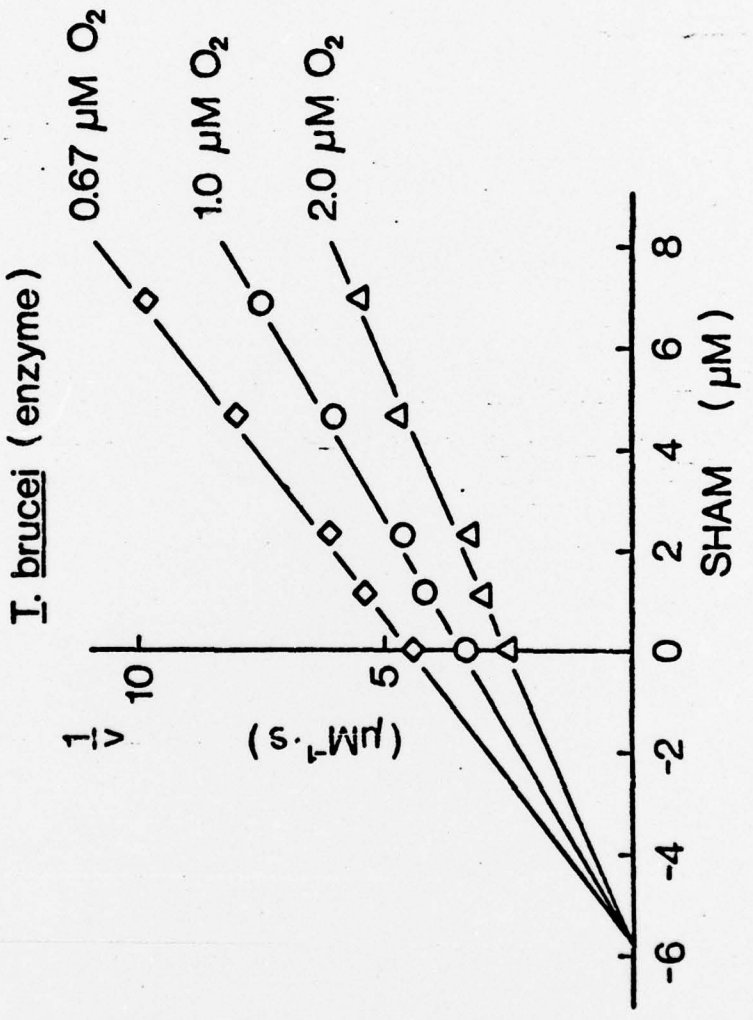

T. brucei (enzyme)
0.67 μM O2
1.0 μM O2
2.0 μM O2
1/v
(μM⁻¹·s)
10
5
-6
-4
-2
0
2
4
6
8
SHAM (μM)

Figure 16

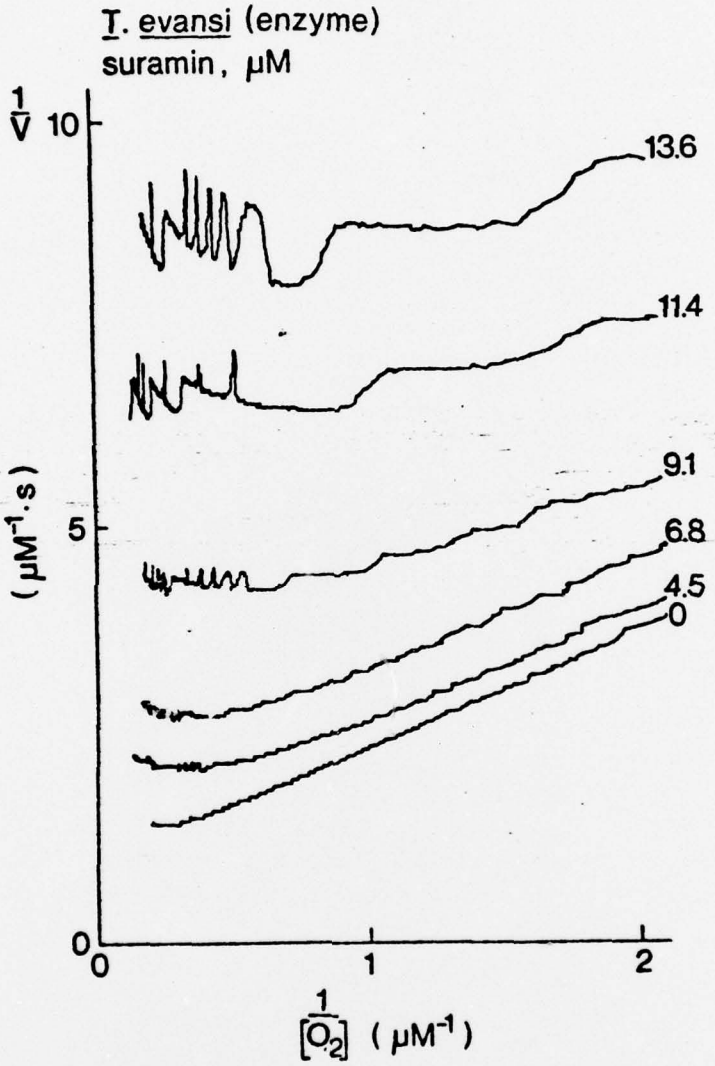

T. evansi (enzyme)
suramin, μM
1/V
10
5
0
(μM⁻¹·s)
13.6
11.4
9.1
6.8
4.5
0
0
1
2
1/[O2] (μM⁻¹)

Figure 17

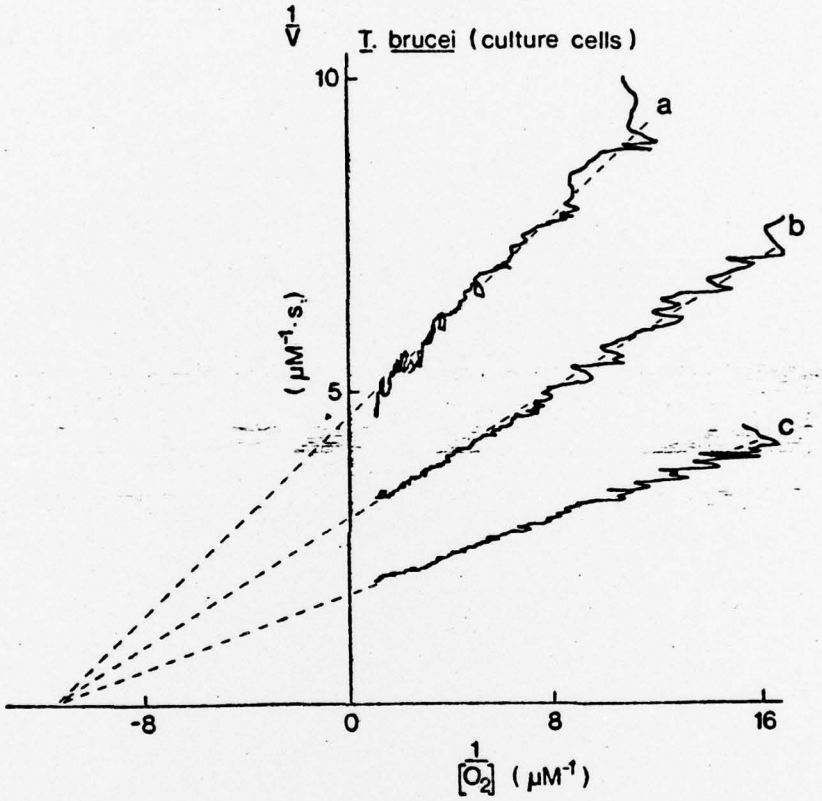
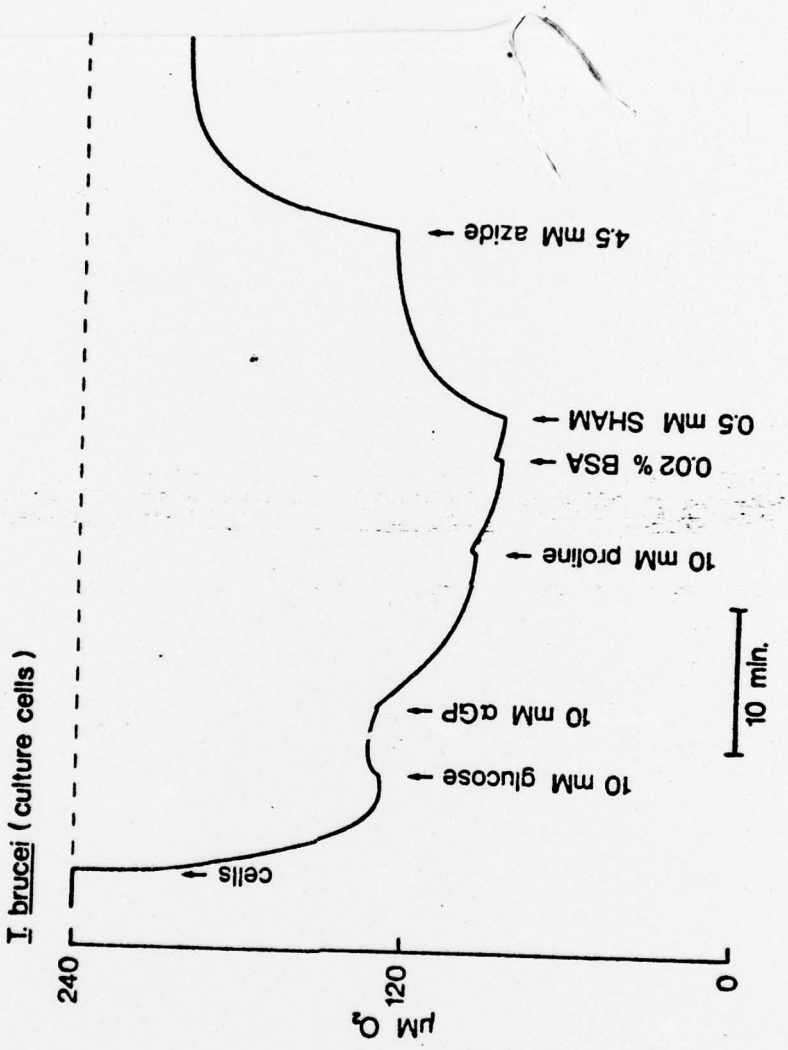


Figure 18


I. brucei (culture cells)
240
120
0
μM O2
cells
10 mM glucose
10 mM αGP
10 mM proline
0.02 % BSA
0.5 mM SHAM
4.5 mM azide
10 min.

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